AD_____

Award Number:
W81XWH-08-1-0398

TITLE:
Coordination of BRCA1/BARD1- and MRE11/RAD50/NBS1-dependent DNA transactions in breast tumor suppression

PRINCIPAL INVESTIGATOR:
Dr. Jean Gautier Ph.D. Dr. Eric Greene Ph.D.

CONTRACTING ORGANIZATION:
Columbia University, New York, NY 10032

REPORT DATE:
July 2009

TYPE OF REPORT:
Annual

PREPARED FOR:
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

   X Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

Report Documentation Page

Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

1. REPORT DATE **01 JUL 2009**	2. REPORT TYPE	3. DATES COVERED **01-08-2008 to 31-07-2009**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER **W81XWH-08-1-0398**
Coordination of BRCA1/BARD1- and MRE11/RAD50/NBS1-dependent DNA transactions in breast tumor suppression	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Columbia University,New York,NY,10032**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**11**	

Table of Contents

INTRODUCTION

BRCA1/BARD1 and MRE11/RAD50/NBS1 (MRN) play critical roles in preventing the onset of breast tumorigenesis. This is underscored by the fact that mutations in BRCA1 are associated with the most frequent form of hereditary breast cancer (*1*) and women who inherit mutations in the *BRCA1* gene have an estimated lifetime risk of breast and/or ovarian carcinoma as high as 85% (*2*). In addition, mutations in NBS1, RAD50 and Mre11 are associated with increased risk for breast cancer (*3, 4*) or with sporadic breast tumors (*5*). Finally, BRCA1/BARD1 and MRN associate in to form DNA damage-specific complexes, critical for damage checkpoint signaling (*6*). To better understand the functional significances of these interactions, we propose to determine how BRCA1/BARD1 and the MRN complex cooperate in the recognition, signaling and repair of DNA damage during DNA replication and at double-stranded breaks (DSBs) induced by DNA damaging agents. We hypothesize that each protein complex influences the behavior of the other on DNA and that the roles of these proteins in the maintenance of genomic stability are ultimately dictated by their dynamic interactions on DNA. The overall objective of this collaborative effort is to understand precisely how BRCA1/BARD1 breast tumor suppressor complex orchestrates DNA transactions critical for genome stability and how this process interfaces with MRN's diverse roles.

BODY

Significant progress has been made towards Task 1 and Task 2, as outlined in the Statement of Work.

In the First Task we proposed to start characterizing BRCA1/BARD1 interactions with DNA by Total Internal Reflection Fluorescent Microscopy (TIRFM).

We have expressed recombinant BRCA1/BARD1 protein complexes in baculovirus-infected cells. We have optimized the conditions of expression and purification of the tagged complex. An example of Coomassie staining of recombinant BRCA1/BARD1 complex is shown in Figure 1.

Figure 1: **Purification of BRCA1/BARD1 complexes.** BRCA1 and BARD1 proteins were expressed in baculovirus-infected cells (sf9 cells). Following infection, the protein were expressed for 72 hours, the cells where lysed and BRCA1/BARD1 protein complex was purified by affinity according to published protocols. The purified protein complex was processed for gel electrophoresis
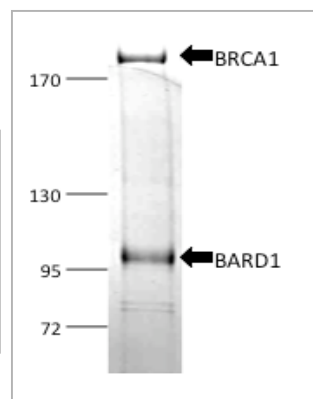
We have begun initial characterization of quantum dot(QD)-tagged Brac1/Bard1 interactions with dsDNA using our single molecule DNA curtain imaging technology. We have shown that the QD labeling strategy works for this protein complex in our single molecule assays, that the labeling is specific, and that the labeled proteins are well-behaved in our experimental system. Furthermore, we have also demonstrated that BRCA1/BARD1 binds double stranded DNA at random locations, and that the protein appears to slide for a short distance along the DNA.

We are currently analyzing the data that we have obtained from both the BRCA1/BARD1 experiments and the MRN experiments (see below) before proceeding to the next phases of the study, which will include the comparative analysis of BRCA1/BARD1 and MRN with the larger complex made up of BRAC1/BARD1/MRN, and we also anticipate beginning work with the mutant proteins as described in the original proposal.

We have established an important new technology in the laboratory that has greatly increased the throughput capacity and reproducibility of our single molecule DNA curtain assays. All of our previous studies relied upon DNA curtains that were assembled at the leading edges of microscale barriers to lipid diffusion. These microscale barriers were made by manually etching the surface of a fused silica sample chamber with a diamond-tipped scribe. Using this approach it was exceedingly difficult to reproducibly generate barriers of similar quality, and it was impossible to make barriers that were located at defined positions on the sample chamber surface. All of these problems led to low data throughput. To solve this, we have established new nanotechnology based methods for making lipid diffusion barriers. These include the use of either electron-beam or nanoimprint lithography, and can be used to make highly reproducible barriers at defined locations, and the barriers themselves have dimensions in the nanometer range. The primary advantage of these new approaches is that we can now image up to one thousand individual DNA molecules in a single field of view, we can attach the DNA in several different configurations, these include molecules anchored by one end and positioned at defined distances from one another (*7, 8*), molecules anchored by two ends, and molecules anchored in criss-crossed configurations.

In the Second Task, we proposed to investigate the interactions between BRCA1/BARD1 and MRN complexes. Analysis by TIRFM and in cell-free extracts.

We have optimized the protocol to transfect insect cells, express baculovirus encoding for the Mre11-Rad50-Nbs1 complex and purify the tagged complex from insect cells lysates. TIRFM experiments require purified protein complex as well as 1:1:1 stoichiometry of the Mre11, Rad50, Nbs1 subunits within the complex. We have found that Nbs1 is the most labile protein of the complex. In preliminary experiments presented for this application, we used tagged Mre11 to purify the MRN complex. While this allow high yield of MRN complexes, we noticed that a fraction of the complexes (variable from experiment to experiments) contained only Mre11 and Rad50 (MR). We have tried different approaches to remedy this potential problem. We have tagged all subunits, using different tags and we have determined that using Tagged Nbs1 (NBS1-FLAG) was optimal. Briefly, the optimized purification protocol is as follow. Hi five cells are seeded in 18x15 cm dishes by passaging confluent cells at a ratio of 1:2 to obtain 2×10^7 cells per dish. Cells are left to attach for 1 hour and were then co-infected with P3 amplified virus

at an MOI of 10 in the following ratio:Nbs1-FLAG:Rad50:Mre11 (5:3:2). Cells are harvested after 72 hrs, washed in 1x PBS and pelleted at 1500 rpm for 4 min. Cells are resuspended in approximately 500ul of buffer containing protease inhibitors per 15 cm dish. Cells are dounced 10 X with an 18 gauge needle and then sonicated 8 x 30 sec. Cells are then put on ice for 15 min before centrifuging in a bench top microfuge at 13,000 rpm for 20 min. <u>Batch purification on Flag beads:</u> The supernatant is bound overnight at 4°C to 300 ul of Anti-Flag M2 agarose beads that has previously been washed 3 times in Magic Buffer. The unbound material is removed from the beads which are subsequently washed 8 times with Magic buffer. MRN complex is eluted with 3x 300µl of 0.25µg/µl 1x Flag peptide.

We have contined to characterize quantum dot(QD)-tagged QD-tagged MRN interactions with dsDNA using our single molecule DNA curtain imaging technology. We have shown that the QD labeling strategy works for this protein complex in our single molecule assays and that the labeling is specific.

We have also shown that MRN slides rapidly along DNA, and that it can bring together two molecules of DNA. This MRN-mediated reaction appears to occur by a zippering mechanism where the molecules first join at their free end, and then are brought together over regions spanning at least 20 to 30 kilobases (kb).

To better understand the interactions between BRCA1/BARD1 and the MRN complex under physiological conditions, we have designed a system to generate DNS double-strand breaks in a chromosomal context and to monitor recruitment of signaling and repair proteins at these chromosomal DSBs. To this end, we incubate demembranated sperm nuclei in cell-free extracts derived from *Xenopus* eggs in the presence of PflMI restriction enzyme (0.05 U/µL). Following enzyme addition, aliquots of the reaction were diluted and centrifuged through a sucrose cushion at the indicated times. Chromatin pellets were then isolated free of cytosol and processed for electrophoresis and Western blotting (Fig. 2). In contrast to other approaches, this method allows us to monitor the behavior of endogenous, untagged proteins in the absence of cross-linking treatment. We observed that Mre11 was constitutively associated with chromatin but was further recruited and modified upon generation of chromosomal DSBs. Next, ATM was recruited to chromatin and simultaneously activated, as seen by the appearance of γH2AX. CtIP was recruited with slower kinetics than ATM, followed by binding of the ssDNA-binding protein RPA, a read-out of DNA

end resection. We found that ATM binding to chromatin peaked early then decreased with time but was never entirely abrogated. Release of ATM from chromatin correlated with RPA recruitment on newly resected DNA, consistent with the idea that ATR activation by ssDNA-RPA intermediates participates in ATM down-regulation (*9*). Similar patterns of sequential chromatin recruitment were seen in S-phase and in M-phase (data not shown). These results are consistent with data in yeast and mammalian cells using GFP-tagged proteins or chromatin immunoprecipitation to analyze sequential recruitment of proteins to DSBs (*10-13*).
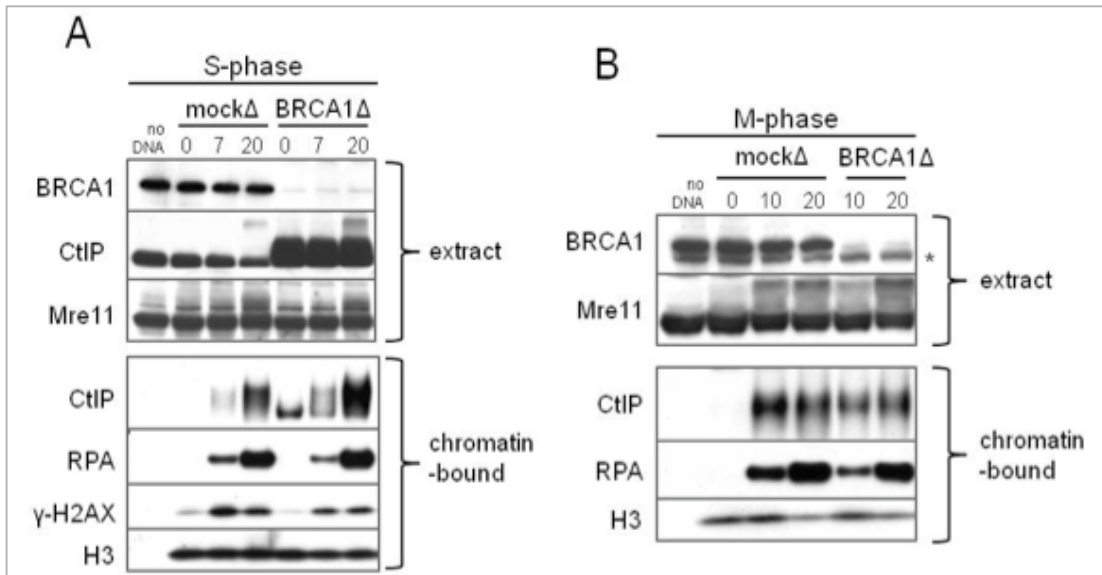
Figure 2. BRCA1 is dispensable for MRN-dependent DNA end resection. A. Control extract (MockΔ), BRCA1 depleted extract supplemented with recombinant CtIP protein (xCtIP/BRCA1Δ) or CtIP depleted extract (CtIPΔ) in S-phase were supplemented with 5,000 nuclei/µl and incubated for 10 minutes at 20°C prior to addition of PmlfI restriction enzyme. Aliquots of the reactions were taken at the indicated times and processed for Western blotting with BRCA1 and CtIP antibodies (extract, top panels). Aliquots of the reaction were taken at the indicated times and chromatin was isolated following centrifugation through a sucrose cushion then processed for eletrophoresis and Western blotting with the indicated antibodies (chromatin-bound, bottom panels). B. Same as (A) with M-phase extracts.

Using this system, we started to evaluate the potential role of BRCA1 in DNA end resection, as seen by the accumulation of RPA on chromatin. BRCA1 interacts with phosphorylated CtIP through its BRCT tandem repeats (*14*), and has been proposed to be required for DNA resection in mammalian cells (*15*). In contrast, loss of BRCA1 in chicken DT40 cells did not inhibit resection (*16*). To test the role of BRCA1 in this system, we generated antibodies against xBRCA1 and depleted BRCA1 from our extracts. We observed that BRCA1 depletion co-depleted CtIP. Because CtIP depletion did not significantly decrease in BRCA1 levels, we hypothesized that CtIP co-depletion

8

might be due to non-specific association with immuno-complexes. Therefore, to evaluate the impact of BRCA1 loss on DNA resection, we supplemented BRCA1-depleted extracts with recombinant xCtIP protein. We found that BRCA1 depletion did not affect CtIP recruitment to damaged chromatin or DNA resection in S-phase (Fig. 2A) or in M-phase (Fig. 2B). We conclude that BRCA1 is dispensable for MRN-dependent DNA end resection.

We have also developed a new type of single molecule assay that enables us to generate a de novo break in a curtain of DNA molecules at a defined location, and this assay will allow us to specifically ask whether MRN, and/or BRCA1/BARD1 can be target to DNA breaks via a sliding mechanism. For this assay, DNA molecules (48.5 kb; 13 µm) are biotinylated at both ends, and then aligned along the leading edge of nanofabricated barrier to lipid diffusion. Because the molecules are biotinylated at both ends, they align in a looped or "U-shaped" configuration, where the DNA folds back on itself and appears to be half of its actual length (~6.5 µm). The molecules can then be rapidly cut by injecting a restriction enzyme that cleaves the DNA at a single site close to one of the tethered ends of the DNA. Upon cleavage, the molecules immediately extend to their full lengths, providing a clear readout for generation of the double strand break. We are no using this assay to test how MRN and BRCA1/BARD1, are targeted to the breaks and how they respond when they reach the broken end. To test this mechanism, we will pre-load the proteins on the unbroken DNA, and then induce the break by injection of the restriction enzyme, and then visualize exactly how the proteins move from the dsDNA region to the newly generated DNA end.

KEY RESEARCH ACCOMPLISHMENTS

- Started to characterize the behavior of BRAC1/BARD1 on DNA, using a single molecule approach.
- Optimized protein complex purification for tagged BRCA1/BARD1.
- Optimized protein complex purification for tagged MRN.
- Established that whereas MRN is essential for processing DNA ends at double-strand breaks (DSBs), BRCA1 is dispensable for this function. In contrast both BRCA1 and MRN complex are required for checkpoint activation following DNA damage.

REPORTABLE OUTCOMES

S. Peterson, R. Baer, M. Gottesman and J. Gautier. Cell Cycle Regulation of DNA Double-strand Break Resection. (In revision)

T. Fazio, M. L. Visnapuu, S. Wind, E. C. Greene, *Langmuir* 24, 10524 (Sep 16, 2008).

M. L. Visnapuu, T. Fazio, S. Wind, E. C. Greene, *Langmuir* 24, 11293 (Oct 7, 2008).

CONCLUSION

Our experiments in cell-free extracts have allowed us to separate the functions of the BRCA1/BARD1 and MRN complex. Because both complex have been associated with the development of breast tumors, we hypothesize that their common role in checkpoint signaling might be critical to prevent breast tumor development.

REFERENCES

1. S. V. Hodgson, P. J. Morrison, M. Irving, *Am J Med Genet C Semin Med Genet* **129**, 56 (Aug 15, 2004).
2. M. C. King, J. H. Marks, J. B. Mandell, *Science* **302**, 643 (Oct 24, 2003).
3. K. Heikkinen *et al.*, *Carcinogenesis* **27**, 1593 (Aug, 2006).
4. J. Steffen *et al.*, *Int J Cancer* **119**, 472 (Jul 15, 2006).
5. T. Sjoblom *et al.*, *Science* **314**, 268 (Oct 13, 2006).
6. R. A. Greenberg *et al.*, *Genes Dev* **20**, 34 (Jan 1, 2006).
7. T. Fazio, M. L. Visnapuu, S. Wind, E. C. Greene, *Langmuir* **24**, 10524 (Sep 16, 2008).
8. M. L. Visnapuu, T. Fazio, S. Wind, E. C. Greene, *Langmuir* **24**, 11293 (Oct 7, 2008).
9. B. Shiotani, L. Zou, *Mol Cell* **33**, 547 (Mar 13, 2009).
10. S. Bekker-Jensen *et al.*, *J Cell Biol* **173**, 195 (Apr 24, 2006).
11. M. Lisby, J. H. Barlow, R. C. Burgess, R. Rothstein, *Cell* **118**, 699 (Sep 17, 2004).
12. A. Rodrigue *et al.*, *EMBO J* **25**, 222 (Jan 11, 2006).
13. E. Berkovich, R. J. Monnat, Jr., M. B. Kastan, *Nat Cell Biol* **9**, 683 (Jun, 2007).
14. X. Yu, L. C. Wu, A. M. Bowcock, A. Aronheim, R. Baer, *J Biol Chem* **273**, 25388 (Sep 25, 1998).
15. L. Chen, C. J. Nievera, A. Y. Lee, X. Wu, *J Biol Chem* **283**, 7713 (Mar 21, 2008).
16. G. Y. Zhao *et al.*, *Mol Cell* **25**, 663 (Mar 9, 2007).